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Increasing acetylcholine levels in the hippocampus or entorhinal cortex reverses the impairing effects of septal GABA receptor activation on spontaneous alternation.

By

Aldemar Bas De Groot

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirement for the degree of Master of Science

Division of Neuroscience

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University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled *Increasing acetylcholine levels in the hippocampus or entorhinal cortex reverses the impairing effects of septal GABA receptor activation on spontaneous alternation* submitted by Aldemar Bas De Groot in partial fulfillment of the requirements for the degree of Master of Science.

Abstract

Intra-septal infusions of the gamma-aminobutyric acid (GABA) agonist muscimol impair learning and memory. The present experiments determined whether hippocampal or entorhinal infusions of the acetylcholinesterase inhibitor physostigmine would reverse such impairing effects on spontaneous alternation performance, a measure of spatial working memory. Male Sprague-Dawley rats were given intra-septal infusions of vehicle or muscimol (1 nmol/0.5 μl) combined with unilateral intra-hippocampal or intra-entorhinal infusions of vehicle or physostigmine (10 μg/μl for the hippocampus; 7.5 μg/μl or 1.875 μg/0.25 μl for the entorhinal cortex). Fifteen minutes later, spontaneous alternation performance was assessed. The results indicated that intra-septal infusions of muscimol significantly decreased percent alternation scores. More importantly, intra-hippocampal or intra-entorhinal infusions of physostigmine, at doses that did not influence performance when administered alone, completely reversed the muscimol induced deficit. These findings support the hypothesis that the impairing effects of septal GABAergic activity involve cholinergic processes in the hippocampus and the entorhinal cortex.

Acknowledgments

First of all, I would like to express gratitude to my supervisor, Dr. Marise Parent for her academic instruction, her inspiration, her ability to motivate me and inspire me and for giving me the opportunity to perform research in her laboratory. In addition I would like to thank Dr. Dallas Treit for providing funding and interesting research opportunities in his lab as well as time and advice. I would also like to thank my other committee member, Dr. Andy Greenshaw, for providing his advice and insights as well as the time that he has taken to sit on my committee.

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Abbreviations

ACh	acetylcholine
AChE	acetylcholinesterase
ANOVA	analysis of variance
AP	anterior posterior
cm	centimetre
DV	dorsal ventral
EC	entorhinal cortex
GABA	gamma-aminobutyric acid
i.m.	intra-muscular
i.p.	intra-peritoneal
kg	kilogram
µl	microlitre
µm	micrometre
mg	milligram
ML	medial lateral
mm	millimetre
NAAG	N-acetyl-aspartyl-glutamate
nmol	nanomol
P	probability
s.c.	subcutaneously
S.E.M.	standard error of the mean

VA Virginia

1. Background and introduction

Background

The pathways between the septum and the hippocampus and between the septum and the entorhinal cortex (EC) are involved in learning and memory. The septo-hippocampal projection plays a role in Parkinson's disease and aging (Hornberger et al., 1985; Court and Perry, 1991; Fibiger, 1991; Perry et al., 1992) and both septo-hippocampal and septo-entorhinal cortex projections are involved in Alzheimer's disease (Cotman and Andersen, 1988; Cotman et al., 1990; Court and Perry, 1991; Fibiger, 1991; Cabalka et al., 1992; Perry et al., 1992). Evidence indicates that stimulating the septal gamma-aminobutyric acid (GABA)ergic system impairs memory (McNamara and Skelten, 1995; Stackman and Walsh, 1995; Herzog et al., 2000) whereas stimulating the cholinergic system of the hippocampus improves memory performance (Ohno and Watanabe, 1996). Similarly, removing the cholinergic input to the EC results in a memory deficit (Walsh et al., 1996). The goal of the present research was to examine the interaction between the GABAergic system in the medial septum and the cholinergic systems in the entorhinal cortex and the hippocampus in learning and memory. We hypothesized that the GABAergic system in the medial septum impairs memory by decreasing extracellular acetylcholine (ACh) levels in the hippocampus and/or the EC. Based on this, we anticipated that the learning and memory deficit produced by stimulating the septal GABAergic system could be reversed by increasing extracellular ACh levels in either structure.

The medial septum.

The septum is part of the septal area, which is, in turn, part of the limbic system (Jakab and Leranth, 1995). Extensive evidence has implicated the medial

septum in learning and memory. Lesions of the medial septum impair acquisition and retention performance in a variety of behavioral paradigms (Mitchell et al., 1982; Bolhuis et al., 1988; Fibiger et al., 1991; M'Harzi and Jarrard, 1992). Furthermore, memory can be enhanced or impaired by intra-septal infusions of drugs that act on a variety of neurotransmitter systems (Brioni et al., 1990; Givens and Olton, 1990; Markowska et al., 1990; Dudchenko and Sarter, 1991).

The septum contains cholinergic and GABAergic cell bodies that project to the hippocampus via the fimbria-fornix (Wainer et al., 1985; Freund and Antal, 1988; Kiss et al., 1990; Nauman et al., 1992). Cholinergic fibers constitute between half and two-thirds of all septo-hippocampal projections, whereas the remaining non-cholinergic neurons are GABA-ergic (Wainer et al., 1985; Freund and Antal, 1988; Kiss et al., 1990; Nauman et al., 1992). These septal fibers reach the hippocampus via the fimbria and the dorsal fornix and terminate mainly in the dentate gyrus and subfields of Ammon's horn (Freund and Antal, 1988). The cholinergic neurons project to the hippocampal pyramidal cells, the dentate granule cells and the inhibitory interneurons (Lewis et al., 1967). The GABAergic neurons, on the other hand, project primarily to the inhibitory interneurons (Babb et al., 1988). The proportion of septal neurons that are either cholinergic or GABAergic is not 100%, and the remaining neurons contain neuropeptides. The neuropeptides that are the most common are galanin and N-acetyl-aspartyl-glutamate (NAAG; Andersen et al., 1961; Melander et al., 1985; Forloni et al., 1987; Senut et al., 1989). Some neuropeptides are colocalized with the cholinergic or GABAergic neurons. Galanin, for instance, is

colocalized with cholinergic neurons and parvalbumin with GABAergic neurons (Melander et al., 1985).

The cholinergic and GABAergic neurons in the medial septum contain both cholinergic and GABAergic receptors (Bialowas and Frotscher, 1987). In addition, they express receptors for other neurotransmitters, such as glutamate (Leranth and Frotscher, 1989). Cholinergic agonists as well as GABAergic antagonists increase the activity of neurons in the medial septum. Conversely, cholinergic antagonists and GABAergic agonists decrease the activity of these neurons (Lamour et al., 1984). Similarly, cholinergic agonists and GABAergic antagonists enhance memory, whereas cholinergic antagonists or GABA agonists impair memory. For instance, infusions of the cholinergic agonist oxotremorine into the septum enhance choice accuracy in aged rats engaged in a memory task (Markowska et al., 1990). On the other hand, septal infusions of the cholinergic antagonist scopolamine or the GABAergic agonist muscimol impair memory in a number of tasks: rewarded alternation, radial arm maze, place discrimination in a Morris water maze, and visual discrimination (Brioni et al., 1990; Givens and Olton, 1990; Dudchenko and Sarter, 1991; Chrobak and Napier 1992;).

Medial septal effects involve the hippocampus.

Evidence indicates that the effects of the medial septum on learning and memory involve the hippocampus (Gray and McNaughton, 1982). Lesions of the septo-hippocampal pathway (i.e. the fimbria fornix) impair learning and memory (Nilsson et al., 1987; Aggleton et al., 1992; Dickinson-Anson et al., 1998). Furthermore, lesions of the medial septum or the fimbria-fornix eliminate hippocampal

theta rhythm, an effect that is associated with memory deficits (Bland, 1986; Dutar et al., 1995; Givens, 1995).

More specifically, the role of the medial septum in learning and memory appears to involve an influence on cholinergic processes in the hippocampus (Brioni et al., 1990; Durkin, 1992; Farr et al., 1999; Herzog et al., 2000). Lesions of the septo-hippocampal pathway, which impair learning and memory, decrease acetylcholinesterase (AChE) staining intensity (Erb et al., 1997) and extracellular acetylcholine (ACh) levels (Nilsson et al., 1990). Hippocampal grafts of ACh-producing cells attenuate the memory impairing effects of fimbria-fornix lesions (Dunnett et al., 1982; Nilsson and Björklund, 1992; Dickinson-Anson et al., 1998). Furthermore, in some instances, IgG-saporin-induced lesions of the cholinergic septo-hippocampal projection impair spatial working memory and decrease hippocampal high affinity choline uptake (Berger-Sweeney et al., 1994; Dornan et al., 1996; Walsh et al., 1996; but see Baxter et al., 1995; Bannon et al., 1996). Thusfar, the reasons for the inconsistent effects of IgG-saporin-induced lesions on learning and memory are unclear. Task demands, type of motivation and method of administration of IgG-saporin do not appear to account for the discrepancies (Chappell et al., 1998; McMahon et al., 1997; Walsh et al., 1996; Pang and Nocera, 1998).

Intra-septal infusions of muscimol impair memory by decreasing extracellular ACh levels in the hippocampus.

Like the effects of septal lesions, the memory-modulating effects of intra-septal drug infusions also appear to involve cholinergic processes in the hippocampus. Intra-septal infusions of the cholinergic antagonist scopolamine, at doses that impair

learning and memory, decrease extracellular cholinergic levels in the hippocampus (Gorman et al., 1994). Administration of benzodiazepines into the medial septum decrease hippocampal ACh levels (Imperato et al., 1993, 1994; Herzog et al., 2000) and impair learning and memory (McNamara and Skelten, 1995; Stackman and Walsh, 1995; Herzog et al., 2000).

Evidence suggests that activation of the GABAergic system in the medial septum also impairs learning and memory by decreasing extracellular cholinergic levels in the hippocampus (Brioni et al., 1990; Durkin, 1992). The effect of muscimol on extracellular ACh levels parallel the action of this compound on memory (Chrobak et al., 1989; Brioni et al., 1990; Givens and Olton, 1990; Dudchenko and Sarter, 1991; Farr et al., 1999). Gorman and colleagues (1994) examined the effects of infusing drugs into the medial septum, at doses that affect memory, on extracellular ACh levels in the hippocampus. They found that the GABA receptor agonist muscimol decreased extracellular ACh levels. Brioni and colleagues (1990) injected various doses of muscimol into the medial septum and measured high affinity choline uptake in the hippocampus, as well as performance on a spatial memory task. They found that doses of muscimol that significantly decreased high affinity choline uptake in the hippocampus also significantly impaired memory performance. Doses that did not affect memory did not affect high affinity choline uptake. Numerous other studies report that infusions of muscimol into the medial septum reduce high affinity choline uptake, as well as the ACh turnover rate and extracellular ACh levels in the hippocampus (Costa et al., 1983; Wood, 1986; Durkin, 1992; Walsh et al., 1993; Gorman et al., 1994; Moor et al., 1998). Further, intra-septal infusions of muscimol

prevent the increase in hippocampal ACh induced by performance in a memory task (Durkin, 1992; Moor et al., 1998).

Purpose of Experiment 1

The evidence indicating that the septal GABAergic system influences memory via an influence on the hippocampal cholinergic system is extensive, but primarily indirect. That is, the effects of intra-septal infusions of muscimol on hippocampal cholinergic measures and memory have been examined in different groups of animals or in the same animal on separate occasions. Septal infusions of the GABA agonist muscimol impair memory (Chrobak et al., 1989; Brioni et al., 1990; Durkin, 1992; Givens and Olton, 1990, 1994; Nagahara and McGaugh, 1992; Nagahara et al., 1992; Wan et al., 1995; Farr et al., 1999), infusions of muscimol in the septum decrease extracellular ACh levels in the hippocampus (Allen and Crawford, 1984; Blaker et al., 1986; Richter and Gormly, 1986; Brioni et al., 1990; Durkin 1992; Walsh et al., 1993; Giovannini et al., 1994; Gorman et al., 1994), and decreases in extracellular ACh levels in the hippocampus are associated with impaired memory (Brioni et al., 1990; Walsh et al., 1996; Herzog et al., 2000). Experiment 1 examined the effects of simultaneously manipulating both the septal GABAergic system and the hippocampal cholinergic system in a rat performing in a learning and memory task. Specifically, the experiment determined whether intra-hippocampal infusions of the AChE inhibitor physostigmine would reverse the impairing effects of intra-septal infusions of the GABA agonist muscimol on spontaneous alternation. We anticipated that physostigmine would increase extracellular ACh levels in the hippocampus and

hypothesized that this would prevent the memory impairments induced by intra-septal infusions of muscimol.

The septo-entorhinal projection.

In addition to sending a cholinergic projection to the hippocampus, the septum also sends a cholinergic projection to the EC (Alanso and Köhler, 1984). The majority of the efferent projections from the septum terminate in layers II and IV (Alonso and Köhler, 1984). Evidence indicates that the EC is involved in many types of memory (Thompson, 1976; Ferreira et al., 1992; Squire, 1992; Wiig and Bilkey, 1994; Suzuki, 1996). For instance, electrolytic, radiofrequency, or aspiration lesions of the EC impair spatial and emotional memory (Steward et al., 1977; Morris et al., 1982; Schenk and Morris, 1985; Rasmussen et al., 1989; Ferreira et al., 1992; Hunt et al., 1994; Johnson and Kesner, 1994;). Infusions of GABA agonists and glutamate antagonists into the EC impair emotional reference memory (Ferreira et al., 1992; Jerusalinsky et al., 1994; Quillfeldt et al., 1994). Infusions of the cholinergic neurotoxin IgG-saporin in the medial septum decrease cholinergic indices in the EC and impair performance in a working memory task (radial arm maze; Walsh et al., 1996).

Purpose of Experiments 2 and 3

The finding that lesions of the cholinergic projection from the medial septum to the EC impair radial arm maze performance (Walsh et al., 1996) suggests that the medial septum may influence memory via a process that also involves an effect on the ACh system in the EC. Therefore, Experiments 2 and 3 determined whether infusions of physostigmine into the EC would reverse deficits in memory produced by intra-

septal infusions of muscimol. It was hypothesized that infusions of physostigmine into the EC would reverse the memory impairment caused by septal GABA receptor activation. In Experiment 2, a relatively large volume of physostigmine was infused into the EC in order to determine if it was possible to obtain a physostigmine effect in the EC. However, this large volume of physostigmine could possibly diffuse to nearby structures such as the ventral hippocampus, a region in the brain that also receives a cholinergic projection from the medial septum (Milner et al., 1983; Jakab and Leranth, 1995). As a result, once an effect was observed in Experiment 2, a smaller volume of physostigmine was used in Experiment 3.

Experiment 1

The purpose of this experiment was to determine whether intra-hippocampal infusions of the AChE inhibitor physostigmine would reverse the impairing effects of intra-septal infusions of the GABA agonist muscimol on spontaneous alternation.

Methods

Subjects

Male Sprague Dawley rats, weighing 250-300g upon arrival, were used. They were individually housed and maintained on a 12 hour light-dark cycle with food and water *ad libitum*. All rats were handled 2 days prior to surgery for 3 minutes each. During handling the rats were given an oral administration of water in order to habituate them to the oral administration of an analgesic on the day of surgery.

Surgery

Surgery was performed at least one week after the rats arrived. On the day of surgery, the rats were given an oral administration of the analgesic acetaminophen (Tylenol 120 mg/1.5 cc). One hour later, they were given atropine sulfate (0.2 cc, i.p.) to reduce respiratory complications produced by the anaesthetic. Subsequently, rats were anaesthetized with pentobarbitol (Nembutal 50 mg/kg, i.p.), hydrated with saline (3 cc, s.c.), and given the antibiotic penicillin (Crystiben, Rhone Merieux Canada Inc., 0.05 cc, i.m.). Stereotaxic surgical procedures were used to implant one 22 gauge stainless-steel guide cannula (Plastics One, Inc. Roanoke, VA) aimed at the medial septum (0.5 mm anterior to bregma [AP], 4.9 mm ventral to dura [DV], 3.2 mm from the interaural line; Paxinos and Watson, 1986) and one guide cannula aimed at the dorsal hippocampus (-4.2 mm AP, 2.0 mm DV, 4.1 mm lateral [ML] to the midline). For half the rats, the hippocampal cannula was implanted in the left hemisphere. The cannulae were attached to the skull with 4 jeweler's screws and cranioplastic cement. A dummy cannula was inserted into each guide cannula to keep the cannula tract clear. Immediately after surgery the rats were placed into a warm environment until they regained consciousness. Two days after surgery, each cannula was checked for obstructions and betadine was applied to the surgical wound.

Procedure and drugs

Two days prior to behavioral testing, all rats were handled for 3 minutes and then handled for 5 minutes the next day. All testing occurred at least one week following surgery between 0900 and 1900 h. Rats were given an infusion of vehicle (phosphate buffered saline, pH 7.4) or physostigmine (10 μg/1 μl/ minute) into the

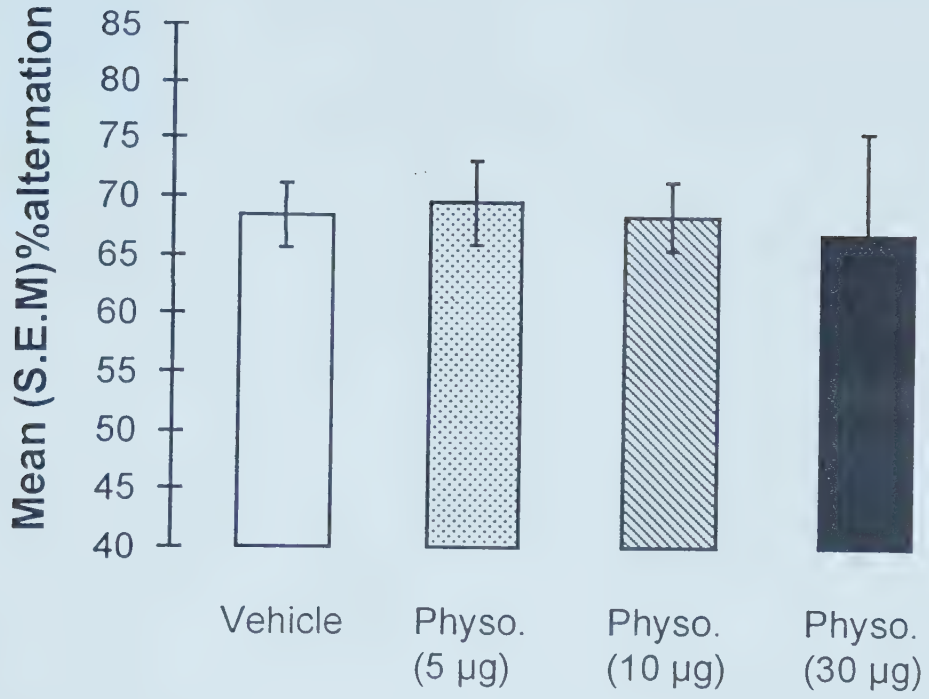
hippocampal cannula, followed immediately by an infusion of phosphate buffered saline or muscimol (1 nmol/0.5 µl/ minute) into the medial septal cannula. During infusions, the experimenter was blind to the drug treatment administered to the rat. The solutions were infused through a 28 gauge injection needle that extended 1 mm beyond the guide cannula. The needle was connected to a 10 µl Hamilton syringe by polyethylene tubing and the infusions were delivered using an infusion pump (Harvard Apparatus 22). The injection needles were left in place for 1 minute following the infusions in order to allow for diffusion. The dose of physostigmine was selected based on pilot experiments indicating that this was the highest dose that did not affect spontaneous alternation performance when infused alone into the dorsal hippocampus (see Figure 1). The muscimol dose was selected based on previous work (Chrobak et al., 1989; Brioni et al., 1990; Parent et al., 1997). The muscimol effect in spontaneous alternation that was obtained by Parent et al., 1997 was successfully replicated prior to commencing Experiment 1 (see Figure 2). Spontaneous alternation performance was tested 15 minutes after the intracranial infusions.

Spontaneous alternation

Evidence suggests that spontaneous alternation is a measure of spatial working memory. In order to alternate between spatial locations, a rat must remember its previous location. A spatial working memory component is supported by evidence that indicates that alternation scores are lowered by the removal of directional cues (Richman et al, 1987) or by increasing the temporal interval between location choices (Livesay et al., 1981; Hepler et al., 1985; Beninger et al., 1986; Shannon et al., 1990; Kelsey and Vargas, 1993; Ragozzino et al., 1996). Furthermore, evidence suggests

Figure 1. A) Infusions of physostigmine (5, 10 or 30 μg/1 μl) into the dorsal hippocampus have no effect on spontaneous alternation performance ($p > .05$ vs. Vehicle/Vehicle; n = 7 - 10 rats per group). B) The effects of the infusions of the number of arms the rats entered in the maze do not parallel the effects of the infusions on percent alternation scores. Infusions of the highest dose (30 μg) of physostigmine into the hippocampus reduce the number of arms the rats entered in the maze.

A


Mean (S.E.M)%alternation
85
80
75
70
65
60
55
50
45
40
Vehicle
Physo. (5 µg)
Physo. (10 µg)
Physo. (30 µg)

B

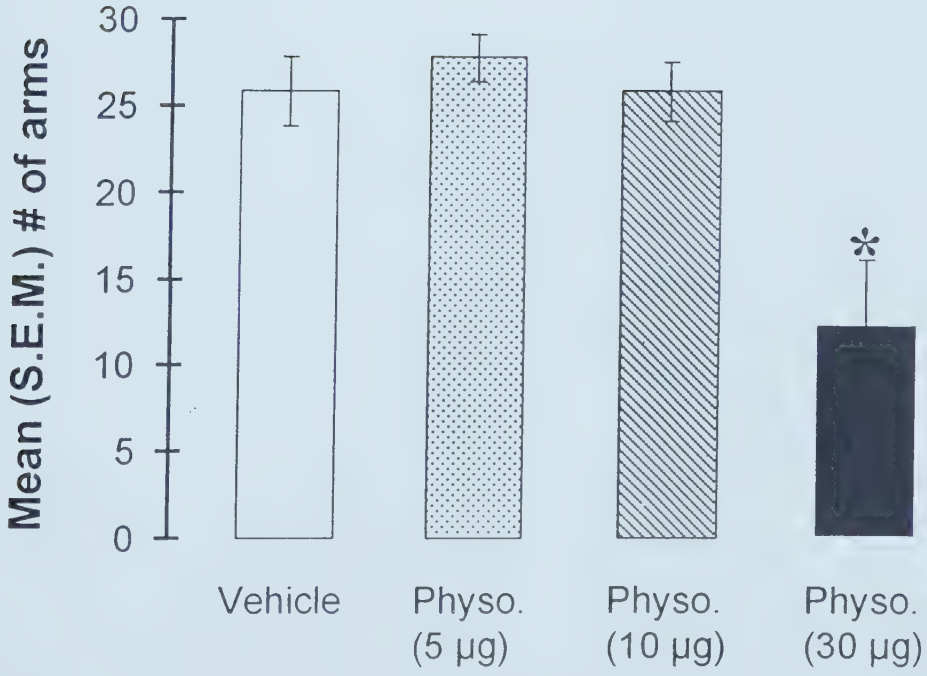
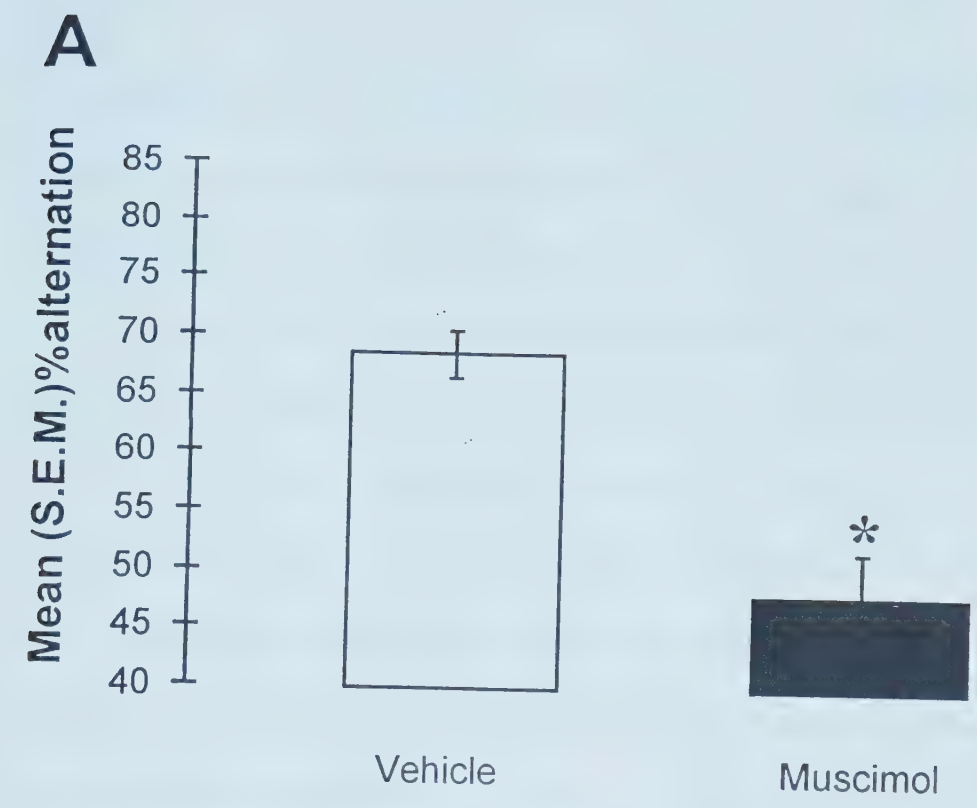
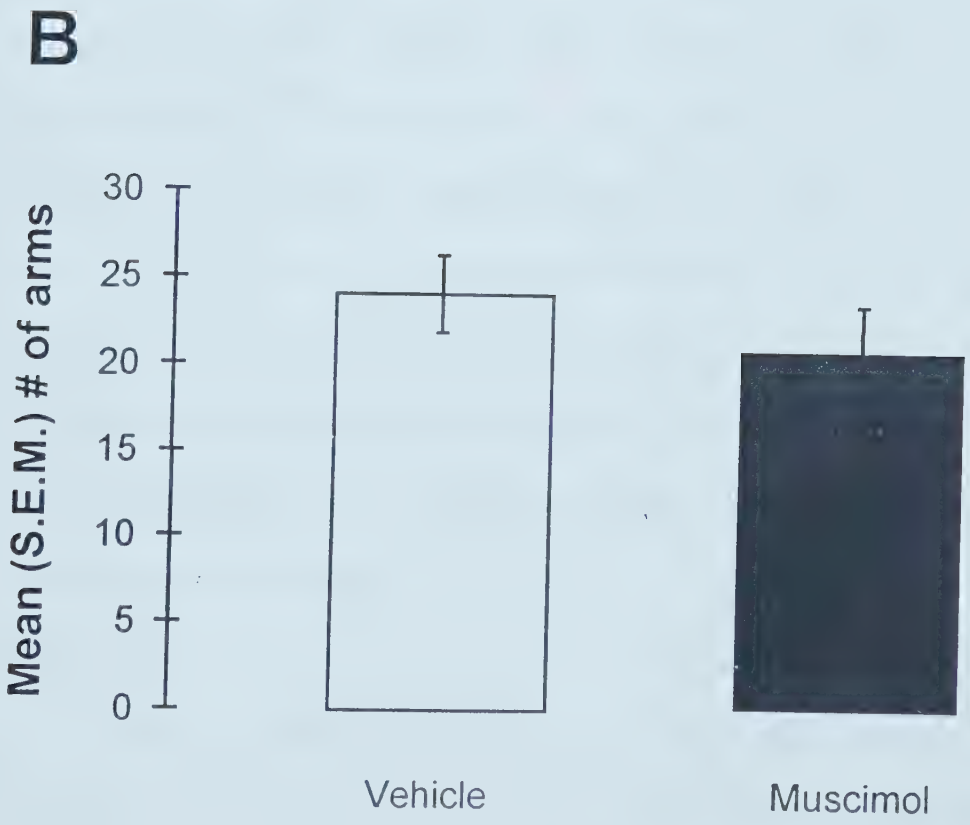

Mean (S.E.M.) # of arms
30
25
20
15
10
5
0
*
Vehicle
Physo. (5 µg)
Physo. (10 µg)
Physo. (30 µg)

Figure 2. A) Infusions of muscimol (114 ng/0.5 µl) into the medial septum significantly impair spontaneous alternation performance ($p < .05$ vs. Vehicle/Vehicle; n = 7 - 10 rats per group). B) Intra-septal infusions of muscimol do not affect the number of arms the rats entered in the maze.


A
Mean (S.E.M.)%alternation
85
80
75
70
65
60
55
50
45
40
*
Vehicle
Muscimol


B
Mean (S.E.M.) # of arms
30
25
20
15
10
5
0
Vehicle
Muscimol

that the effects of septal manipulations on spontaneous alternation are similar to the effects of these manipulations on other measures of spatial working memory, such as rewarded alternation, alternation in the water maze, and radial arm maze (Chrobak et al., 1989; Brioni et al., 1990; Givens & Olton, 1990; Durkin, 1992; Nagahara & McGaugh, 1992; Nagahara et al., 1992; Givens & Olton, 1994; Markowska, et al., 1995; Wan et al., 1995). Spontaneous alternation has the advantage over these tasks in that food-deprivation or immersion in water is not required to motivate the rats.

Spontaneous alternation performance was assessed using a Y-maze composed of three equally spaced arms (120°; 60 cm long x 17.5 cm high). The floor of each arm consisted of stainless steel (3.5 cm wide) and each arm was covered with a translucent Plexiglas lid. Each rat was placed in one arm of the maze and allowed to explore the maze for 8 minutes, and the number of arms and the sequence of the arms entered were recorded. An alternation consisted of entering three different arms consecutively. A percent alternation score was calculated for all rats that entered 10 or more arms in the maze. This score was computed by dividing the number of alternations each rat made by the number of arms entered minus two. The resulting number was multiplied by 100. Data collected for rats that entered less than 10 arms in the maze was not included.

Histology

After the completion of the behavioral tests, the rats were euthanized with an overdose of chloral hydrate and perfused intracardially with 0.9% saline followed by 10% formalin. The brains were removed and placed in a 10% formalin solution. At least 48 hours later, the brains were frozen and sectioned (60 μm), mounted onto glass

slides, and stained with thionin. The cannula location for each rat was determined using a microscope by an observer blind to the drug treatment and the behavioral results. All of the injections had to occur within the medial septum and the dorsal hippocampus (Experiment 1) or the medial septum and the EC (Experiments 2 and 3). The data of those animals whose cannulae were misplaced were not included in the statistical analyses. In addition, data from rats whose histology showed neuronal damage were discarded. In the case of ambiguity, the histology was examined by an additional observer who was also blind to the behavioral data and the drug treatment, and who had sufficient experience to make an informed decision.

Statistics

The spontaneous alternation data were expressed as means and standard errors of the mean (S.E.M.) and analyzed using analysis of variance (ANOVA) and Fisher protected LSD post hoc tests. An alpha level of .05 was used as the criterion for statistical significance.

Results

Figure 3 shows the location of a representative cannula in (A) the medial septum and (B) the dorsal hippocampus. The data from 12 rats was excluded due to misplaced cannulae. Of these 12 rats, four rats received infusions of vehicle in both the hippocampus and the medial septum, seven rats were infused with physostigmine in the hippocampus and vehicle in the medial septum, and one rat received an infusion of physostigmine in the hippocampus combined with muscimol in the medial septum. Further, the data from two rats was excluded because of neuronal damage. One of these rats was infused with physostigmine in the hippocampus and vehicle in the

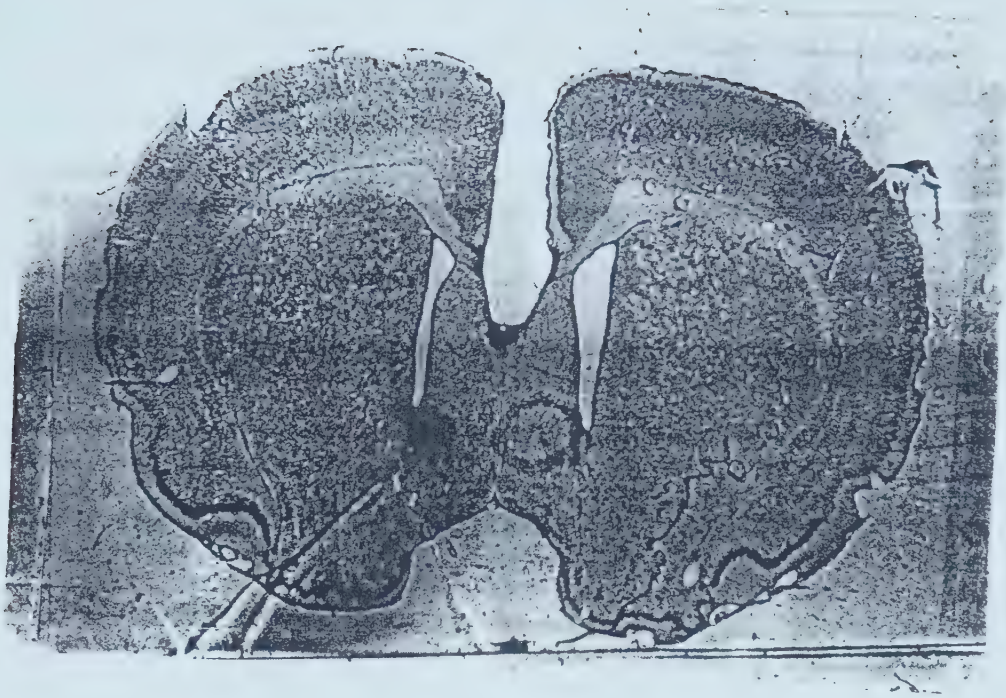
medial septum, while the other was administered an infusion of physostigmine in the hippocampus and an infusion of muscimol in the medial septum. Fifteen rats entered an insufficient number of arms. Seven of these rats were infused with vehicle in the hippocampus combined with muscimol in the medial septum. The remaining eight rats received infusions of physostigmine in the hippocampus and muscimol in the medial septum. Figure 4 shows the distribution of all infusion sites in the (A) medial septum and (B) dorsal hippocampus. The results indicated that the infusions into the hippocampus and the septum significantly affected spontaneous alternation performance [$F(3, 36) = 7.94$; $p < .001$; see Figure 5A]. As expected, intra-septal infusions of muscimol impaired spontaneous alternation performance. The percent alternation scores of rats given vehicle into the hippocampus and muscimol into the septum were significantly lower than those of rats given infusions of vehicle into both regions ($p < 0.5$). Infusions of physostigmine into the hippocampus did not affect spontaneous alternation performance. The percent alternation scores of rats given infusions of physostigmine into the hippocampus and vehicle into the septum were not significantly different from those of rats given infusions of vehicle into both regions ($p > .05$). Importantly, infusions of physostigmine into the hippocampus reversed the impairing effects of the intra-septal infusions of muscimol. The percent alternation scores of rats given infusions of physostigmine into the hippocampus and infusions of muscimol into the septum were significantly higher than those of rats given concurrent infusions of vehicle into the hippocampus and muscimol into the septum ($p < .05$), but were not different from those of rats given infusions of vehicle into both regions ($p > .05$).

The infusions also affected the number of arms the rats entered in the maze [$F(3, 36) = 4.8$; $p < .01$; see Figure 5B]. Rats given infusions of physostigmine into the hippocampus combined with infusions of muscimol into the septum entered less arms than did rats given infusions of vehicle into both regions ($p < .05$). However, rats given infusions of physostigmine into the hippocampus and vehicle into the septum, and rats given vehicle into the hippocampus and muscimol into the septum did not significantly differ from rats given infusions of vehicle into both regions ($p > .05$).

The effects of the infusions on the number of arms the rats entered did not mirror the effects of the infusions on spontaneous alternation. There was no significant correlation between spontaneous alternation performance and the number of arms the rats entered in the maze [$r(38) = .09$; $p > .05$].

Figure 3. Coronal brain sections stained with thionin indicating representative locations of A) the medial septal cannulae (0.70 mm from Bregma) and B) the dorsal hippocampal cannulae (-4.80 mm from Bregma).

A



B

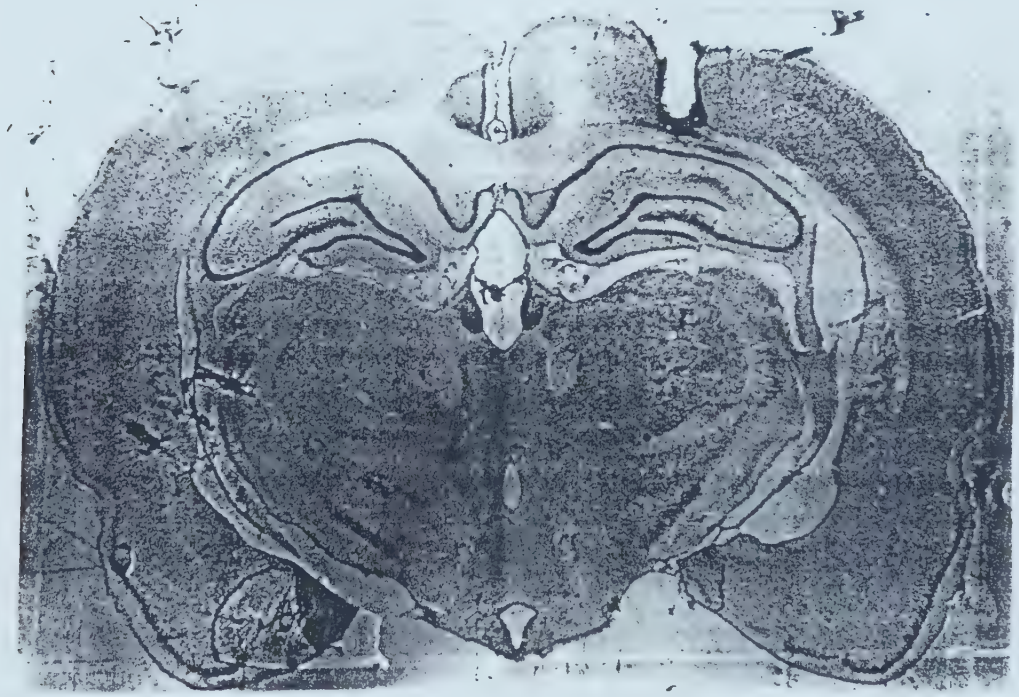


Figure 4. Schematic illustration of a coronal section of the rat brain showing the approximate location of A) medial septal and B) hippocampal infusion sites in Experiment 1. The numbers indicate the position relative to Bregma in mm. Atlas plates adapted from Paxinos and Watson (1986).

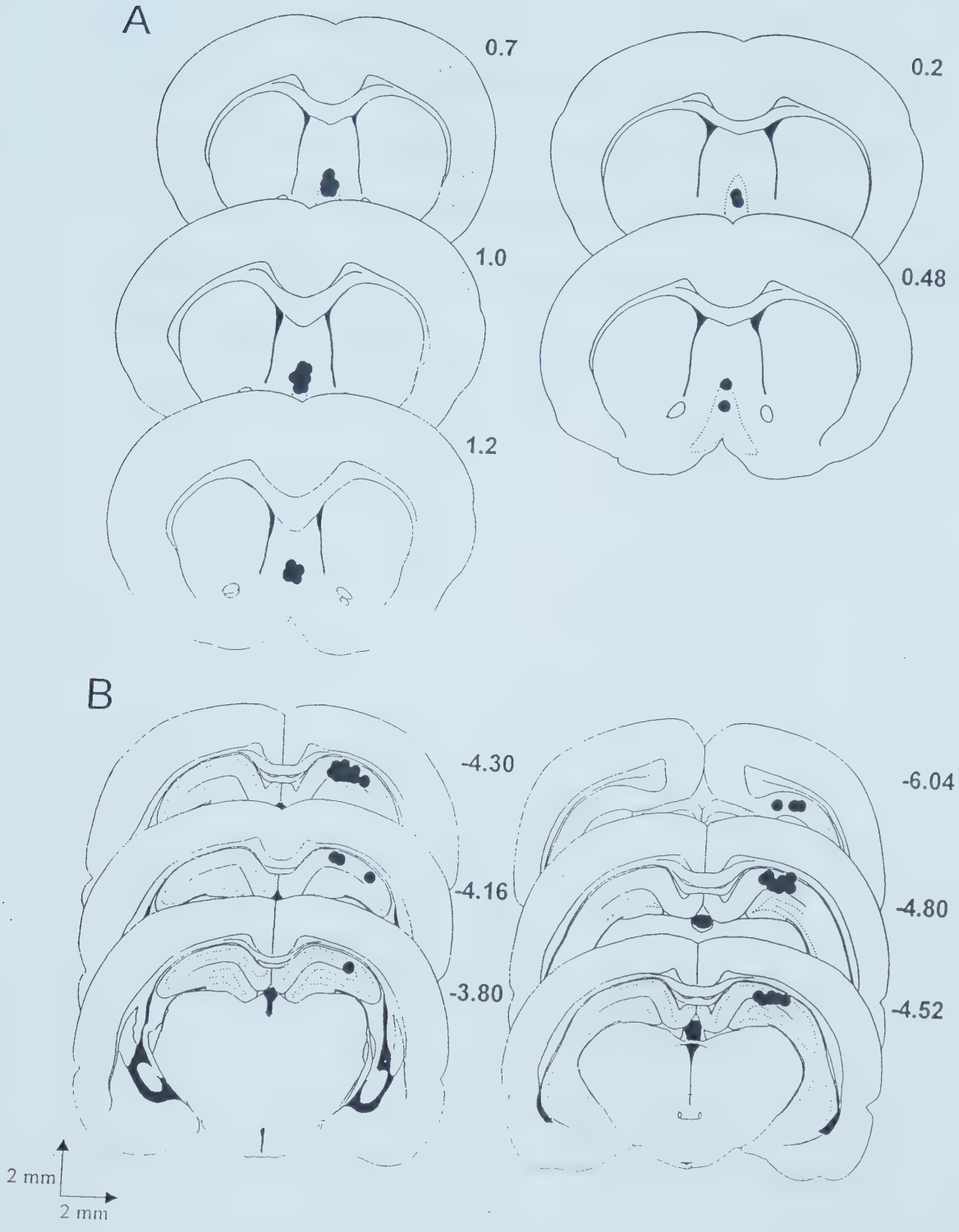
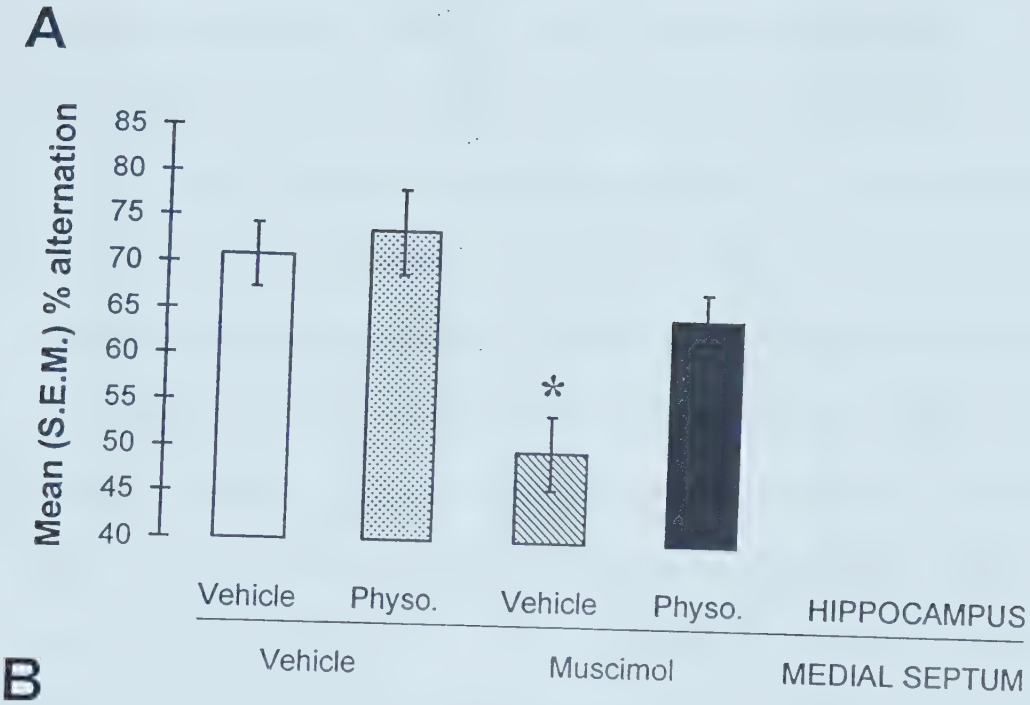
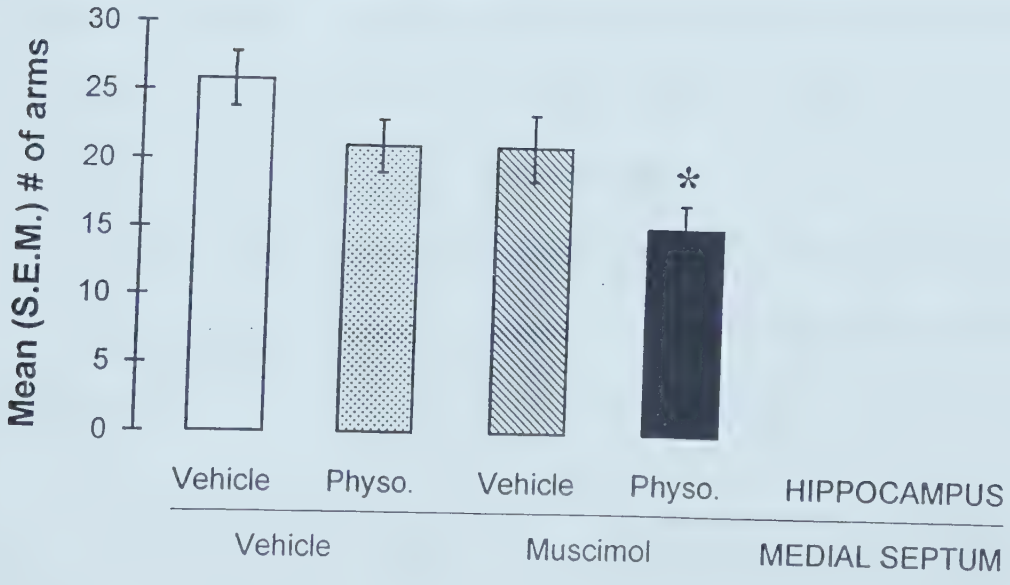

A
0.7
1.0
1.2
0.2
0.48
B
-4.30
-4.16
-3.80
-6.04
-4.80
-4.52
2 mm
2 mm

Figure 5. A) Infusions of physostigmine (10 µg/1 µl) into the dorsal hippocampus reverse the impairing effects of intra-septal infusions of muscimol (114 ng/0.5 µl) on spontaneous alternation performance (* $p < .05$ vs. all other groups; n = 8 - 11 rats per group). B) The effects of the infusions of the number of arms the rats entered in the maze do not parallel the effects of the infusions on percent alternation scores. Concurrent infusions of physostigmine into the hippocampus and muscimol in the medial septum significantly reduce the number of arms the rats entered in the maze (* $p < .05$ vs Vehicle/Vehicle).


A
Mean (S.E.M.) % alternation
85
80
75
70
65
60
55
50
45
40
*
Vehicle
Physo.
Vehicle
Physo.
HIPPOCAMPUS
Vehicle
Muscimol
MEDIAL SEPTUM


B
Mean (S.E.M.) # of arms
30
25
20
15
10
5
0
*
Vehicle
Physo.
Vehicle
Physo.
HIPPOCAMPUS
Vehicle
Muscimol
MEDIAL SEPTUM

Discussion

The present results demonstrate that infusions of the AChE inhibitor physostigmine into the dorsal hippocampus reverse the impairing effect of muscimol infusions into the medial septum on spontaneous alternation performance. These findings support the hypothesis that the impairing effects of intra-septal infusions of muscimol involve cholinergic processes in the hippocampus. The effects of the drugs on spontaneous alternation were not likely related to changes in locomotor activity, as the effects of the drugs on the number of arms the rats entered in the maze and percent alternation were not significantly correlated. For example, muscimol impaired alternation performance without affecting the number of arms the rats entered in the maze. The simultaneous infusion of physostigmine and muscimol did significantly decrease the number of arms the rats entered in the maze. However, this finding does not likely account for the ability of physostigmine to reverse the muscimol-induced deficit. A decrease in arm entries would likely lengthen the latency between arm choices and increase the mnemonic demands of the task (Dember, 1989).

Experiment 2

The purpose of Experiment 2 was to determine whether an infusion of physostigmine into the EC would reverse the impairing effects of intra-septal infusions of muscimol.

Methods

The procedure used in this experiment was the same as that used in Experiment 1 with the following exceptions:

Surgery

Stereotaxic surgical procedures were used to implant one guide cannula aimed at the medial septum and one aimed at the EC (-7.5 mm AP, 6.2 mm DV, 5.3 mm ML). The unilateral EC cannulae were counterbalanced for hemisphere.

Procedure

The rats were given an infusion of vehicle or physostigmine (7.5 μg/1 μl/ minute) into the EC cannula immediately followed by an infusion of vehicle or muscimol (1 nmol/0.5 μl/ minute) into the septal cannula. The physostigmine dose was selected based on pilot experiments that showed that this was the most effective dose for reversing the muscimol-induced impairment. Furthermore, this dose did not affect spontaneous alternation performance when infused alone into the EC.

Results

Figure 6 shows the location of a representative cannula in the EC and Figure 7 shows the distribution of the infusion sites in the EC. Infusion sites in the medial septum were similar to those shown in Figure 4. The data from five rats was excluded due to misplaced cannulae. Two of these rats were infused with vehicle in both the EC and the medial septum while the remaining three rats received an infusion of physostigmine in the EC and muscimol in the medial septum. Further, the data from one rat was excluded because of neuronal damage. This rat was administered physostigmine in the EC and vehicle in the medial septum. Fourteen rats entered an insufficient number of arms. Seven of these rats were infused with vehicle in the EC combined with muscimol in the medial septum. The remaining seven rats were administered physostigmine in the EC and muscimol in the medial septum. The medial

septal and EC infusions significantly affected spontaneous alternation performance [$F(3, 20) = 5.23$; $p < .01$; see Figure 8A]. As in Experiment 1, the infusions of muscimol into the septum significantly impaired spontaneous alternation performance. The percent alternation scores of rats given vehicle into the EC and muscimol into the septum were significantly lower than those of rats given infusions of vehicle into both regions ($p < .05$). Infusions of physostigmine into the EC did not affect spontaneous alternation performance. The percent alternation scores of rats given infusions of physostigmine into the EC and vehicle into the septum were not significantly different from those of rats given infusions of vehicle into both regions ($p > .05$). However, infusions of physostigmine into the EC reversed the impairing effects of intra-septal infusions of muscimol. The percent alternation scores of rats given infusions of physostigmine into the EC combined with infusions of muscimol into the septum were significantly higher than those of rats given vehicle into the EC and muscimol into the septum ($p < .05$), but were not different from those of rats given infusions of vehicle into both regions ($p > .05$).

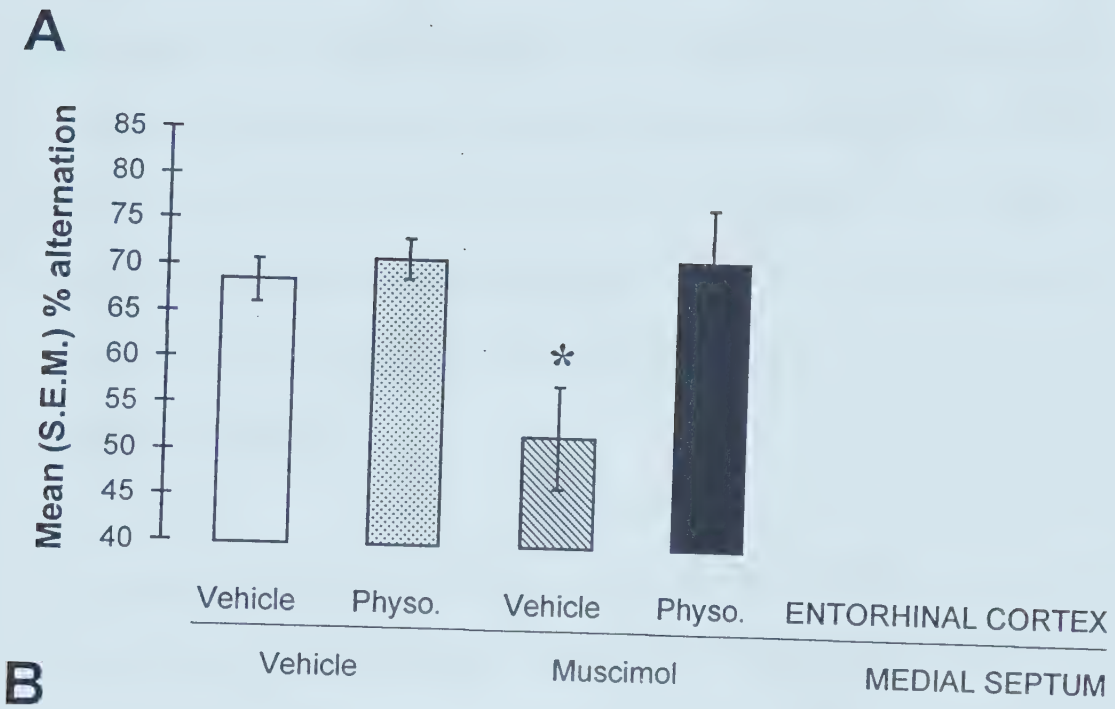
Figure 8B illustrates that the infusions did not affect the number of arms the rats entered in the maze [$F(3, 20) = 0.13$; $p > .05$].

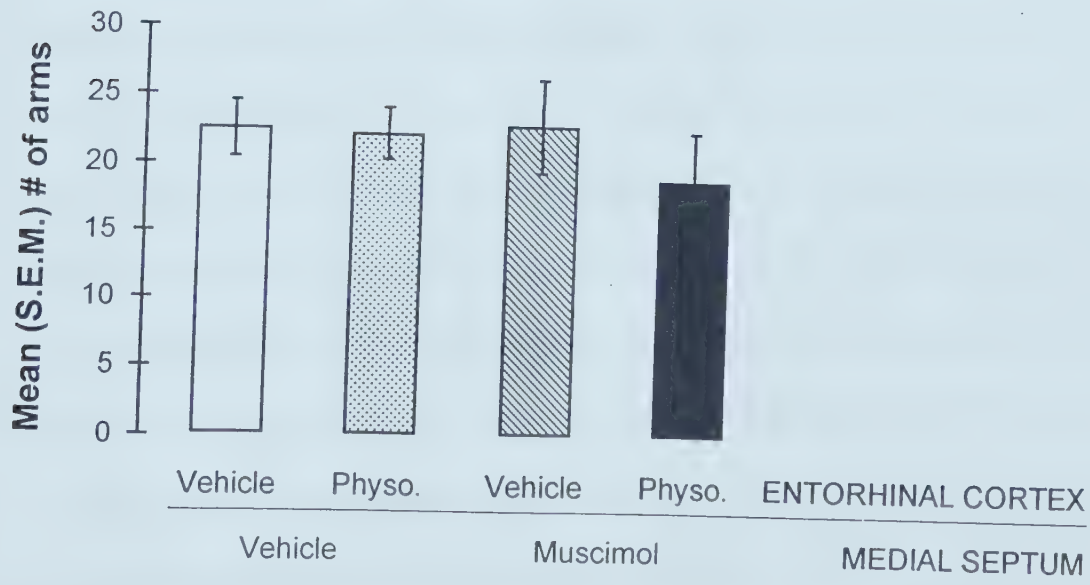
Figure 6. Coronal brain section stained with thionin indicating the representative location of cannulae in the EC (-7.04 mm from Bregma).

Figure 7. Schematic illustration of a coronal section of the rat brain showing the approximate location of EC infusion sites in Experiment 2. The numbers indicate the position relative to Bregma in mm. Atlas plates adapted from Paxinos and Watson (1986).


-7.04
-6.80
-6.72
1 mm
1 mm

Figure 8. A) Infusions of physostigmine (7.5 µg/1 µl) into the EC reverse the impairing effects of intra-septal infusions of muscimol on spontaneous alternation performance (* $p < .05$ vs all other groups; n = 5 - 6 rats per group). B) The infusions do not affect the number of arms the rats entered in the maze ($p > .05$).


A
Mean (S.E.M.) % alternation
85
80
75
70
65
60
55
50
45
40
*
Vehicle
Physo.
Vehicle
Physo.
ENTORHINAL CORTEX
Vehicle
Muscimol
MEDIAL SEPTUM


B
Mean (S.E.M.) # of arms
30
25
20
15
10
5
0
Vehicle
Physo.
Vehicle
Physo.
ENTORHINAL CORTEX
Vehicle
Muscimol
MEDIAL SEPTUM

Discussion

The findings of Experiment 2 demonstrate that, like the effects of infusions of physostigmine into the hippocampus, infusions of physostigmine into the EC reverse the impairing effects of intra-septal infusions of muscimol on spontaneous alternation performance. These findings raise the possibility that the impairing effects of medial septal GABA receptor activation may also involve the cholinergic system in the EC. The effects of muscimol and physostigmine do not appear to be related to changes in locomotor activity, as the infusions did not significantly affect the number of arms the rats entered in the maze.

Experiment 3

Although the findings of Experiment 2 suggest that the GABAergic system of the medial septum and the cholinergic system of the EC interact in spontaneous alternation, the possibility remains that the relatively large volume (1 µl) of physostigmine influenced performance by diffusing to proximal structures. For example, physostigmine could have affected cholinergic processes in the ventral hippocampus, a neural region that also receives a cholinergic projection from the medial septum (Milner et al., 1983; Jakab and Leranth, 1995). A relatively large volume was initially employed in order to first determine if it was possible to obtain an effect of physostigmine in the EC. However, to more clearly implicate cholinergic processes in the EC, Experiment 3 examined the effects of infusing a smaller volume (0.25 µl) of the same concentration of physostigmine into the EC.

Methods

The procedure used in this experiment was the same as that used in Experiment 2 with the following exceptions:

Procedure

Rats were given an infusion of vehicle or physostigmine (1.875 μg/0.25 μl/ minute) into the entorhinal cannula immediately followed by an infusion of vehicle or muscimol (1 nmol/0.5 μl/ minute) into the septal cannula.

Results

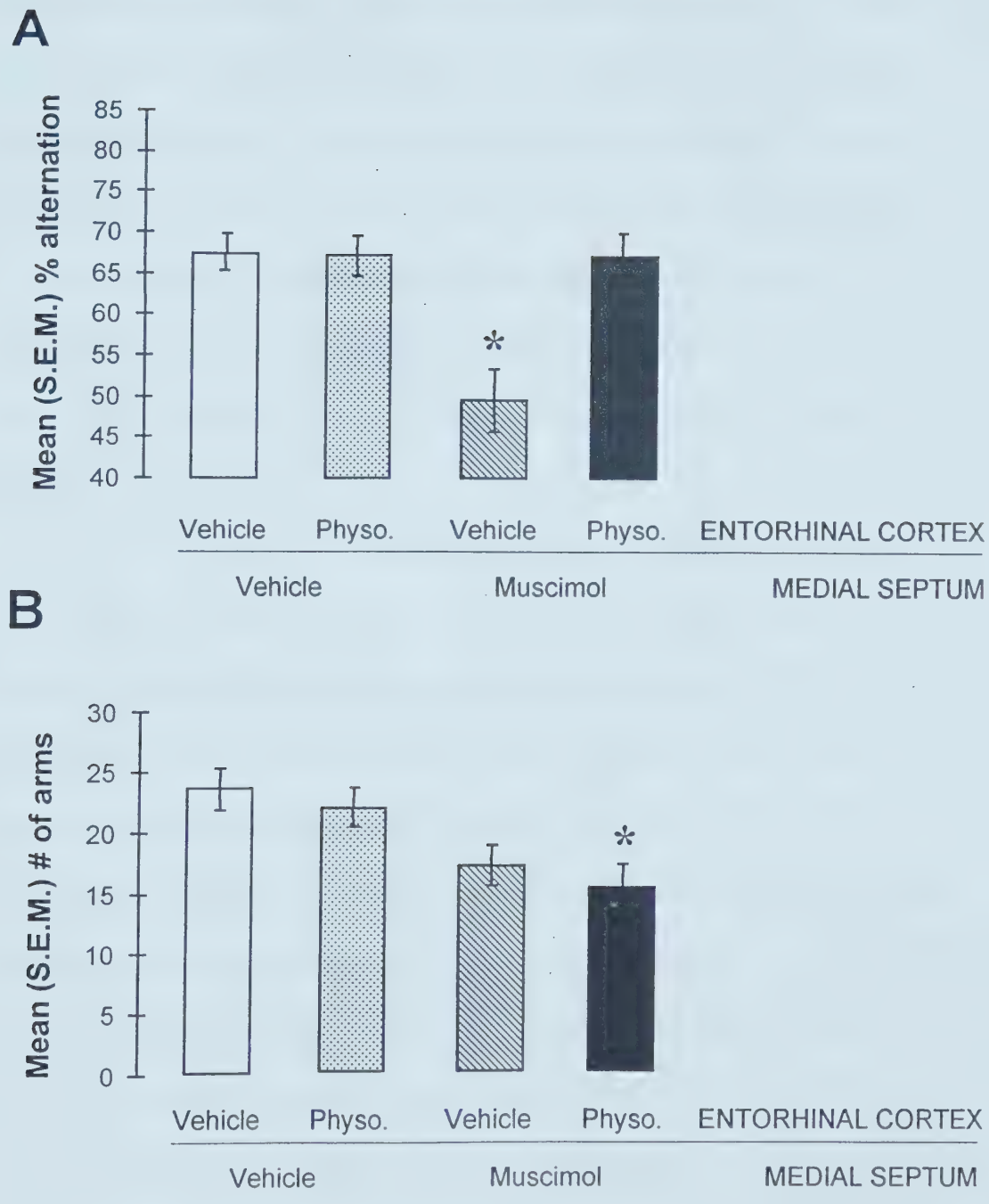
Septal and EC infusion sites were similar to those shown in Figures 4 and 7, respectively. The data from 10 rats was excluded due to misplaced cannulae. Of these 10 rats, two rats were infused with vehicle in both the EC and the medial septum, one rat was administered physostigmine in the EC and vehicle in the medial septum, four rats received infusions of vehicle in the EC and muscimol in the medial septum, and three rats received an infusion of physostigmine in the EC and muscimol in the medial septum. Further, the data from four rats was excluded because of neuronal damage. Two of these rats were infused with vehicle in the EC and muscimol in the medial septum, while the remaining two were administered physostigmine in the EC and muscimol in the medial septum. Fifteen rats entered an insufficient number of arms. Eight of these rats were infused with vehicle in the EC combined with muscimol in the medial septum. The remaining seven rats received infusions of physostigmine in the EC and muscimol in the medial septum. As in Experiment 2, the medial septal and EC infusions significantly affected spontaneous alternation performance [$F(3, 29) = 9.09$; $p < .001$; see Figure 9A]. Infusions of muscimol into the septum significantly impaired

spontaneous alternation performance. The percent alternation scores of rats given vehicle into the EC and muscimol into the septum were significantly lower than those of rats given infusions of vehicle into both regions ($p < .05$). Infusions of physostigmine into the EC did not affect spontaneous alternation performance. The percent alternation scores of rats given infusions of physostigmine into the EC and vehicle into the septum were not significantly different from those of rats given infusions of vehicle into both regions ($p > .05$). Like the effects of the larger volume (1 μl) of physostigmine used in Experiment 2, the results of Experiment 3 indicated that infusions of a smaller volume (0.25 μl) of physostigmine into the EC reversed the impairing effects of the intra-septal infusions of muscimol. The percent alternation scores of rats given infusions of physostigmine into the EC combined with infusions of muscimol into the septum were significantly higher than those of rats given vehicle into the EC and muscimol into the septum ($p < .05$), but were not different from those of rats given infusions of vehicle into both regions ($p > .05$).

Figure 9B indicates that the infusions also affected the number of arms the rats entered in the maze [$F(3, 29) = 4.77$; $p < .01$]. Specifically, rats given infusions of physostigmine into the EC combined with infusions of muscimol into the septum entered less arms than did rats given infusions of vehicle into both regions ($p < .05$). However, rats given infusions of physostigmine into the EC and vehicle into the septum and rats given vehicle into the EC and muscimol into the septum did not significantly differ from rats given infusions of vehicle into both regions ($p > .05$). The effects of the infusions on the number of arms entered were not paralleled by the effects of the infusions on spontaneous alternation. There was no significant

correlation between spontaneous alternation performance and the number of arms the rats entered in the maze [$r(31) = .19; p > .05$].

Figure 9. A) Infusions of physostigmine (1.875 µg/0.25 µl) into the EC in a volume smaller than that used in Experiment 2 also reverse the impairing effects of intra-septal infusions of muscimol on spontaneous alternation performance (* $p < .05$ vs all other groups; n = 7 - 10 rats per group). B) The effects of the infusions of the number of arms the rats entered in the maze do not parallel the effects of the infusions on percent alternation scores. Concurrent infusions of physostigmine into the EC and muscimol in the medial septum significantly reduce the number of arms the rats entered in the maze (* $p < .05$ vs Vehicle/Vehicle).


A
Mean (S.E.M.) % alternation
85
80
75
70
65
60
55
50
45
40
*
Vehicle
Physo.
Vehicle
Physo.
ENTORHINAL CORTEX
Vehicle
Muscimol
MEDIAL SEPTUM
B
Mean (S.E.M.) # of arms
30
25
20
15
10
5
0
*
Vehicle
Physo.
Vehicle
Physo.
ENTORHINAL CORTEX
Vehicle
Muscimol
MEDIAL SEPTUM

Discussion

The findings of Experiment 3 demonstrate that, like the effects of a 1 µl infusion, infusions of a smaller volume of physostigmine (0.25 µl) into the EC also block the impairing effects of intra-septal infusions of muscimol on spontaneous alternation performance. These findings suggest that the medial septal GABAergic system may impair memory via a process that involves the cholinergic system in the EC. The effects of the infusions on spontaneous alternation do not appear to be related to changes in locomotor activity, as the effects of the infusions on percent alternation and the number of arms the rats entered in the maze were not significantly correlated.

General Discussion

The present results indicate that up-regulating cholinergic levels in the hippocampus or the EC completely reverse spontaneous alternation deficits produced by stimulating the medial septal GABAergic system. Specifically, the findings of Experiment 1 indicate that intra-hippocampal infusions of the AChE inhibitor physostigmine fully reverse the impairing effects of intra-septal infusions of muscimol. Furthermore, the results indicate that this effect does not appear to be associated with drug-induced differences in locomotor activity. Likewise, the results of Experiment 2 and 3 demonstrate that infusions of physostigmine into the EC can also reverse the memory-impairing effects of intra-septal muscimol infusions.

The results of the pilot studies indicate that an increase in cholinergic levels in normal rats is only beneficial in a limited range. That is, too much acetylcholine significantly reduces the number of arms entered by the rat. This is congruent with

other studies that report that systemic injections of high doses of physostigmine result in strong sedation (Silvestre et al., 1999).

In the present experiments, both the septal GABAergic and hippocampal cholinergic neurotransmitter systems were simultaneously manipulated while a rat was exploring a maze. Consequently, in comparison with previous findings, the results of the current study provide more direct evidence to support the hypothesis that stimulating the GABAergic system in the medial septum impairs learning and memory through a process that involves a decrease in ACh levels in the hippocampus (Chrobak et al., 1989; Brioni et al., 1990; Givens and Olton, 1990; Dudchenko and Sarter, 1991; Durkin, 1992; Walsh et al., 1993; Gorman et al., 1994; Moor et al., 1998). The results obtained in Experiment 1 are analogous to those obtained by Parent and colleagues (1997), who found that intra-hippocampal infusions of glucose reversed the impairing effects of intra-septal infusions of muscimol. Intra-hippocampal glucose perfusions increase hippocampal cholinergic levels when the cholinergic system is challenged (Ragozzino et al., 1998). Combined with the present results, these latter findings suggest that intra-hippocampal infusions of glucose may have reversed the muscimol-induced deficit by increasing hippocampal ACh levels.

Intra-septal infusions of muscimol likely influence memory via an effect on GABAergic receptors present on both cholinergic and GABAergic septo-hippocampal projection neurons (Van der Zee and Luiten, 1994; Gao et al., 1995; Henderson, 1995; Levey et al., 1995). This possibility is suggested by the finding that lower doses of muscimol are required to impair spatial working memory when the cholinergic projection is lesioned with IgG-saporin (Pang and Nocera, 1998). Combined with

these findings, the present findings suggest that increasing ACh levels in the hippocampus is sufficient to overcome deficits produced by impairing both pathways.

To our knowledge, our findings are the first to suggest that the septal GABAergic system may also influence memory via an effect on cholinergic processes in the EC. Our findings are consistent with those of Walsh and colleagues (1996), who found that lesions of the cholinergic projection from the septum to the EC resulted in a significant loss in cholinergic indices in the EC, as well as a memory deficit in a variable-delay radial-arm maze task. We are confident that the effects of physostigmine resulted from a specific effect on synapses in the EC. First, we show that small volumes of physostigmine (0.25 μl) are as effective as larger volumes (1 μl). Second, an analysis of the data obtained from rats with misplaced cannula (data not shown) indicate that the drug effect is specific to the EC. Although the sample size is small (n = 2), our results indicate that physostigmine did not reverse muscimol-induced deficits when the infusions were too dorsal.

The present findings do not indicate whether the EC mediates its effect on spontaneous alternation via its connection to the hippocampus or whether it acts independently of the hippocampus. The EC provides a major excitatory input to the hippocampus, known as the perforant path (Gauthier and Destrade, 1984). Iijama and colleagues (1996) used real time imaging to examine entorhinal-hippocampal interactions. They showed that neuronal activity was transferred in a frequency dependent manner from the EC to the hippocampus. The authors concluded that the EC is involved in memory by gating the entry of information into the hippocampus. This interpretation is supported by the finding that hippocampal, but not EC lesions

impair performance in a radial arm maze (Jarrard et al., 1984). Alternatively, there is evidence suggesting that the EC plays a distinct role in memory processes that is independent of the hippocampus. For example, stimulation of the EC improves retention in an appetitive learning task in rats with bilateral perforant path lesions (Gauthier and Destrade, 1984). Future experiments will need to determine whether intra-entorhinal infusions of physostigmine can reverse the impairing effects of septal GABA receptor activation when the perforant path is lesioned. If the EC functions through its interaction with the hippocampus, then increasing cholinergic levels in the EC should not reverse a muscimol-induced deficit when the perforant path is lesioned.

It is possible that muscimol ultimately impairs memory by decreasing glutamate levels in the hippocampus. Cholinergic neurons synapse onto glutamatergic cells in the hippocampus (Moor et al., 1996). The individual roles of glutamatergic and cholinergic processes in the hippocampus have been well-demonstrated (Izquierdo et al., 1993; Staubli et al., 1994; Ohno and Watanabe, 1996). Evidence suggests that the glutamatergic and cholinergic systems in the hippocampus interact during learning and memory. Ineffective doses of a cholinergic antagonist and a glutamatergic antagonist significantly impair inhibitory avoidance (Ohno and Watanabe, 1996) and visual recognition memory (Matsuoka and Aigner, 1996) when the two are co-infused. Further, ACh amplifies N-methyl-D-aspartate receptor-mediated responses in a hippocampal slice (Markram and Segal, 1990). These combined findings raise the possibility that the physostigmine-induced up-regulation of cholinergic levels in the hippocampus might reverse the impairing effects of intra-septal infusions of muscimol

by increasing glutamatergic levels. Future experiments will need to examine whether intra-hippocampal infusions of glutamate will reverse the impairing effects of septal GABA receptor activation. In addition, experiments will also be needed to determine whether increasing cholinergic levels in the ventral hippocampus would also reverse deficits induced by intra-septal infusions of muscimol, because the medial septum also sends a cholinergic projection to this area (Milner et al., 1983: Jakab and Leranth, 1995).

In summary, the present findings demonstrate that infusions of the AChE inhibitor physostigmine into the hippocampus or the EC completely reverse the impairing effects of intra-septal infusions of the GABA agonist muscimol on spontaneous alternation. These findings suggest that the septal GABAergic system may impair memory by down-regulating ACh levels in the hippocampus and the EC. Alterations in the septo-hippocampal pathway are associated with Parkinson's disease and age-related memory deficits (Hornberger et al., 1985; Court and Perry, 1991; Fibiger, 1991; Perry et al., 1992). Both septo-hippocampal and septo-entorhinal projections are involved in Alzheimer's disease (Cotman and Andersen, 1988; Cotman et al., 1990; Court and Perry, 1991; Fibiger, 1991; Cabalka et al., 1992; Perry et al., 1992). Therapy with AChE inhibitors has been shown to slow the advancement of deficits associated with Alzheimer's disease (Knapp et al., 1994). Similarly, the administration of AChE inhibitors is effective in some other neurological deficits that are associated with depressed cholinergic levels such as aging (Myers et al., 1996). Thus, the findings of the present study could provide further insight into the underlying mechanisms involved in these neurological insults, as well as provide information

regarding possible treatments. In addition, pilot studies in the present experiment suggest that while an increase in cholinergic levels can enhance memory performance in rats whose cholinergic system is depressed there is no gain and perhaps even a deficit in normal animals. Clinically, this suggests that individuals with normal acetylcholine levels stand little to gain from cholinergic therapy.

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